Fas/Fas Ligand Mediates Keratinocyte Death in Sunitinib-Induced Hand-Foot Skin Reaction

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Sunitinib, a multitargeted receptor Y kinase inhibitor (TKI) used for the treatment of renal cell carcinoma and gastrointestinal stromal tumor (GIST), is notorious for cutaneous adverse effects, such as hand-foot skin reaction (HFSR). To explore the underlying mechanism of HFSR, we enrolled 53 sunitinib-treated GIST patients, including 23 HFSR cases, and 30 tolerant controls. Among the 29 biomarkers examined, soluble FasL (sFasL) showed significant increase in the plasma, blister fluids, and skin lesions of HFSR patients. The plasma levels of sFasL were significantly correlated with those of sunitinib in HFSR patients. In addition to FasL, augmented expression of Fas and active caspase 3 was also detected in the epidermis of HFSR patients. The increased FasL caused keratinocyte death, as the use of anti-FasL antibody specifically blocked cell apoptosis. Oral administration of sunitinib to mice increased skin susceptibility to mechanical injuries in a dose/time-dependent manner. The administration of sunitinib (40 mg kg^{-1} per day) for 4 weeks to mice caused the maximally affected skin area with an erosion-to-ulceration response to tape-stripping. The skin biopsies of mice administered sunitinib exhibited increased expression of Fas and FasL in the apoptotic keratinocytes in the epidermis. Our data revealed that Fas/FasL interaction mediates keratinocyte death in sunitinib-induced HFSR.

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INTRODUCTION

Sunitinib is a multitargeted receptor Y kinase inhibitor that is used for the treatment of metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST) (Chow and Eckhardt, 2007). Sunitinib inhibits cellular signaling of multiple receptor Y kinases, including all receptors for platelet-derived growth factor (PDGF-Rs), vascular endothelial growth factor receptors (VEGFRs), KIT (CD117), Fms-like Y Kinase-3 (FLT3), colony-stimulating factor 1, and RET proto-oncogene, all of which are involved in a great variety of malignancies (Gan *et al.*, 2009). Despite their effectiveness and benefits on the treatment of cancer and survival, sunitinib and other Y kinase inhibitors (e.g., sorafenib) are notorious for their relatively high frequency of cutaneous adverse reactions, including mucositis (up to 20% of patients), rash (up to 40%), alopecia (up to 27%), xerosis (up to 16%), xerostomia (up to 11%), and hand-foot skin reaction (HFSR) (up to 60%; Adams and Leggas, 2007; Gan *et al.*, 2009; Manchen *et al.*, 2011; Nardone *et al.*, 2012).

HFSR, also known as ''palmar plantar erythrodysesthesia'' or ''chemotherapy-induced acral erythema'', is characterized by thick, hyperkeratotic, and erythema/edema lesions with pain, a loss of feeling, and swelling and redness in the palms of hands and/or soles of feet, which occurs with chemotherapies and emerges with targeted therapies (Lacouture *et al.*, 2008; Anderson *et al.*, 2009; Lipworth *et al.*, 2009). Although HFSR is not life threatening, such an adverse reaction is associated with significant tenderness affecting function and quality of life, which often leads to dose modification or discontinuation of treatment (Chu *et al.*, 2007; George *et al.*, 2009; Lee *et al.*, 2009). Asian patients exhibit increased susceptibility to Y kinase inhibitor-induced HFSR (Anderson *et al.*, 2009; George *et al.*, 2009; Lee *et al.*, 2009; Chen *et al.*, 2011; Lee *et al.*, 2012). Genetic polymorphisms of tumor necrosis factor-α, VEGF, and UGT1A9 genes have been reported to link to HFSR in patients of hepatocellular carcinoma treated with sorafenib (Lee *et al.*, 2013).

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Abbreviations: GIST, gastrointestinal stromal tumor; HFSR, hand-foot skin reaction; sFasL, soluble Fas ligand; VEGF, vascular endothelial growth factor

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Currently, several hypotheses regarding the pathophysiology of HFSR have been suggested. Sunitinib-induced HFSR was proposed to be caused by poor repair of repeated small traumas to the hands and feet due to the inhibition of VEGF receptors and PDGF receptors, or by direct skin toxicity from the drug (Cheng *et al.*, 2009). Accumulation of potentially toxic concentrations of the causative drug in eccrine sweat glands, which are present in greatest number or density in the palms and soles may lead to a direct cytotoxicity against acral epithelium in HFSR (Mrozek-Orlowski *et al.*, 1999; Jacobi *et al.*, 2005). In addition, damaged vascular integrity and keratinocyte injury may have an impact on HFSR induced by anti-angiogenic agents (Tsai *et al.*, 2006; Lacouture *et al.*, 2008). Such theory is supported by the association of VEGF polymorphisms with high-grade HFSR in patients with hepatocellular carcinoma after sorafenib therapy (Lee *et al.*, 2013). Others proposed a more mechanical mechanism in which VEGF inhibition and direct pressure from walking, hand washing, and other daily use combine to damage capillary endothelium and cause the skin damage and blistering observed in HFSR (Tsai *et al.*, 2006). To explore the molecular mechanism of HFSR, in this study, we recruited 53 sunitinib-treated GIST patients, and analyzed potential biomarkers.

RESULTS

Elevated plasma levels of sunitinib in patients with HFSR

We enrolled 53 GIST patients (32 males, 21 females) who were imatinib resistant, and then received sunitinib treatment. Patients were followed up after sunitinib administration at regular intervals (median follow-up time: 13.9 months). The adverse reactions induced by sunitinib in GIST patients included diarrhea, mucositis/stomatitis, hypertension, HFSR, rash, and skin discoloration, out of which HFSR was the most common (Table 1). Among 53 GIST patients, 23 (43.4%) developed grade 1–3 HFSR, and the remaining 30 subjects without HFSR were considered as sunitinib-tolerant controls (Table 1). We observed that HFSR usually occurred in the palms or soles of patients who have received the oral administration of sunitinib (37.5 ~ 50 mg per person per day) for 4–8 weeks, which is consistent with the previous reports (Lacouture *et al.*, 2008; Yang *et al.*, 2010). We obtained plasma samples of 17 HFSR patients at the acute stage and 24 tolerant controls at steady-state after 4 weeks of daily dosing. The plasma levels of sunitinib were significantly increased in HFSR patients (mean ± SD: 85.8 ± 27.3 ng ml^{-1}; range: 52.9–130.2) than those of tolerant controls (53.4 ± 14.5 ng ml^{-1}; 19.7–87.1; $P = 0.008$; Figure 1a), suggesting the poor metabolism and delayed clearance of sunitinib in HFSR patients.

Table 1. Adverse events of sunitinib-treated GIST patients

Variable	Sunitinib ($n = 53$)			
Adverse event	Grade 1	Grade 2	Grade 3	Grade 4
Diarrhea	9 (17.0%)	13 (24.5%)	0	0
Mucositis/stomatitis	8 (15.1%)	1(1.9%)	1 (1.9%)	0
Hypertension	5 (9.4%)	9 (17.0%)	5 (9.4%)	0
Hand-foot skin reaction	5 (9.4%)	9 (17.0%)	9 (17.0%)	0
Rash	4 (7.5%)	4 (7.5%)	0	0
Skin discoloration	8 (15.1%)	0	0	0

Abbreviation: GIST, gastrointestinal stromal tumor.

Elevated FasL levels in the plasma and blister fluids of HFSR patients

We examined the plasma levels of 29 potential biomarkers, including 27 cytokines, and 2 cytotoxic proteins (FasL and granulysin) in HFSR patients, tolerant controls, and healthy donors (Figure 1b, Supplementary Figure S1 online). Among the tested factors, soluble Fas ligand (sFasL) showed the most significant increase in the plasma of HFSR patients (mean ± SEM: 119.8 ± 21.0 pg ml^{-1}) compared with tolerant controls (50.4 ± 4.2 pg ml^{-1}; $P = 0.0048$) and healthy donors (53.2 ± 3.7 pg ml^{-1}; $P = 0.0065$) (Figure 1b). No significant difference in the plasma levels of other cytokines, such as IL-6 and tumor necrosis factor-α-α, was detected between HFSR patients and tolerant controls (Supplementary Figure S1a online). Of note, the level of granulysin, a cytotoxic protein identified as a key mediator of keratinocyte death in Stevens–Johnson syndrome and toxic epidermal necrolysis (Chung *et al.*, 2008; Krensky and Clayberger, 2009), showed no difference between HFSR and controls (Supplementary Figure S1b online). The FasL levels in the blister fluids were significantly increased in the HFSR patients ($n = 3$; mean ± SEM: 133.2 ± 18.98 pg ml^{-1}) compared with those in burn patients ($n = 10$; 67.73 ± 10.55 pg ml^{-1}; $P = 0.0216$; Figure 1c). Furthermore, the plasma levels of FasL were correlated with sunitinib ($n = 17$, $r = 0.56$, $P = 0.019$; Figure 1d). These results suggest that FasL may be a biomarker of sunitinib-induced HFSR.

Elevated expression of Fas, FasL, and cleaved caspase 3 in the skin lesions of HFSR patients

The histopathological analyses of HFSR skin biopsies showed hyperkeratosis, acanthosis, and intraepidermal cavities filled with necrotic keratinocytes (Figure 2, Supplementary Figures S2 and S3 online). In addition, loss of keratinocyte cohesion and keratinocyte necrosis was observed at the level of the stratum spinosum/granulosum (Figure 2, Supplementary Figures S2 and S3 online). Compared with the skin samples of healthy donors, increased expression of FasL, Fas receptor, and cleaved caspase 3 was noticed in the keratinocytes around the intraepidermal areas of HFSR (Figure 2, Supplementary Figures S2 and S3 online). Staining with isotype control antibodies showed no immunereactivity in the skin biopsies (Supplementary Figure S3 online). These results reveal the increased expression of Fas, FasL, and active caspase 3 in the keratinocytes of the epidermis in the skin lesions of HFSR.

FasL mediates the cytotoxicity of HFSR plasma to keratinocyte cultures

To investigate FasL-mediated cytotoxicity in HFSR, we cultured human keratinocytes with 10% plasma from HFSR

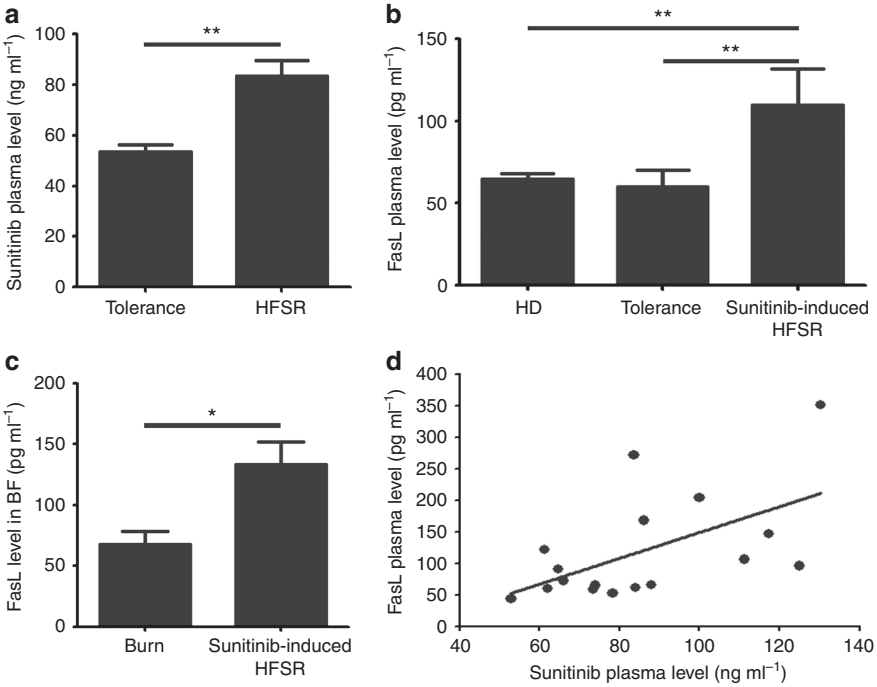

Figure 1. Elevated levels of sunitinib and FasL in the plasma and blister fluids of sunitinib-induced HFSR patients. The plasma levels of sunitinib (**a**) and sFasL (**b**) were analyzed by high-performance liquid chromatographic or ELISA in the samples of tolerant controls (tolerance; $n=14$), sunitinib-induced HFSR patients ($n=17$), and the healthy donors (HD; $n=10$). (**c**) The FasL levels in the blister fluids (BF) of burn patients ($n=10$) and HFSR patients ($n=3$) were measured by ELISA. (**d**) Correlation of plasma levels of FasL and sunitinib in the HFSR patients ($n=17$, $r=0.56$, $P=0.019$). Data are presented as mean ± SEM. $^*P<0.05$; $^{**}P<0.01$.

patients ($n=5$), healthy donors ($n=5$), or 10% fetal bovine serum (medium control) for 24 hours and analyzed the cytotoxicity by the CCK-8 assay. The plasma from HFSR patients significantly induced cytotoxicity to keratinocytes in comparison with the medium control or plasma from healthy subjects (Figure 3a). The increased cytotoxicity was abolished by the anti-FasL antibody (5G51) that could interfere with Fas/FasL interaction (Collins *et al.*, 2005); however, unaffected by isotype control antibody (Figure 3a). To further assess FasL-mediated keratinocyte apoptosis in HFSR, we incubated keratinocytes with 10% plasma from HFSR patients ($n=5$) for 6 hours and stained annexin V. Compared with the medium control or plasma from healthy donors, an augmented apoptotic response was observed in the keratinocytes treated with 10% plasma of HFSR patients (Figure 3b and c). This apoptotic response is specifically diminished by anti-FasL antibody, but not the isotype control antibody (Figure 3b and c), suggesting that FasL mediates keratinocyte apoptosis in HFSR.

Oral administration of sunitinib to mice increased the skin susceptibility to mechanical injuries

Anticancer therapy–induced HFSR has been proposed to involve the combined effects of direct pressure to the affected areas and drug toxicity (Tsai *et al.*, 2006). To develop an animal model of HFSR, we orally administered different doses of sunitinib (0, 20, 40 mg kg^{-1} per day) to mice for various time periods (1, 2, and 4 weeks) and evaluated the skin response to tape-stripping. The tape-stripping method allowed us to induce a mechanical disruption of the epidermal barrier and concurrently study the effect of sunitinib on skin with mechanical injuries. The expression of Fas/FasL was then examined in the back skin with tape-stripping and in the foot pads without tape stripping. In the absence of tape-stripping, no apparent skin injuries or HFSR was found in the mice administered the maximal dose (40 mg kg^{-1} per day) of sunitinib (Figure 4) for 4 weeks. With tape stripping, the mice showed increased susceptibility to mechanical pressure in a sunitinib dose/time-dependent manner (Figure 4). Significant increase of the affected area was found in mice administered sunitinib at 40 mg kg^{-1} per day for 2 weeks ($P=0.0043$), 20 mg kg^{-1} per day for 4 weeks ($P=0.0087$), or 40 mg kg^{-1} per day for 4 weeks ($P=0.0022$; Figure 4). In particular, the mice receiving sunitinib (40 mg kg^{-1} per day) for 4 weeks showed the maximally affected skin areas with an erosion-to-ulceration response to tape-stripping application (Figure 4).

Sunitinib increased the expression of Fas/FasL in the keratinocytes of the epidermis in mice

The skin biopsies of mice were obtained and subjected to hematoxylin and eosin (H&E) staining and immunohistochemistry studies. The *in vivo* efficacy of sunitinib was demonstrated by a decreased level of phosphor-PDGFR (p-PDGFR) in the subcorneal epidermis of foot pads of treated mice (Supplementary Figure S4 online). H&E staining of affected skin samples showed epidermal acanthosis with erosions or ulcers

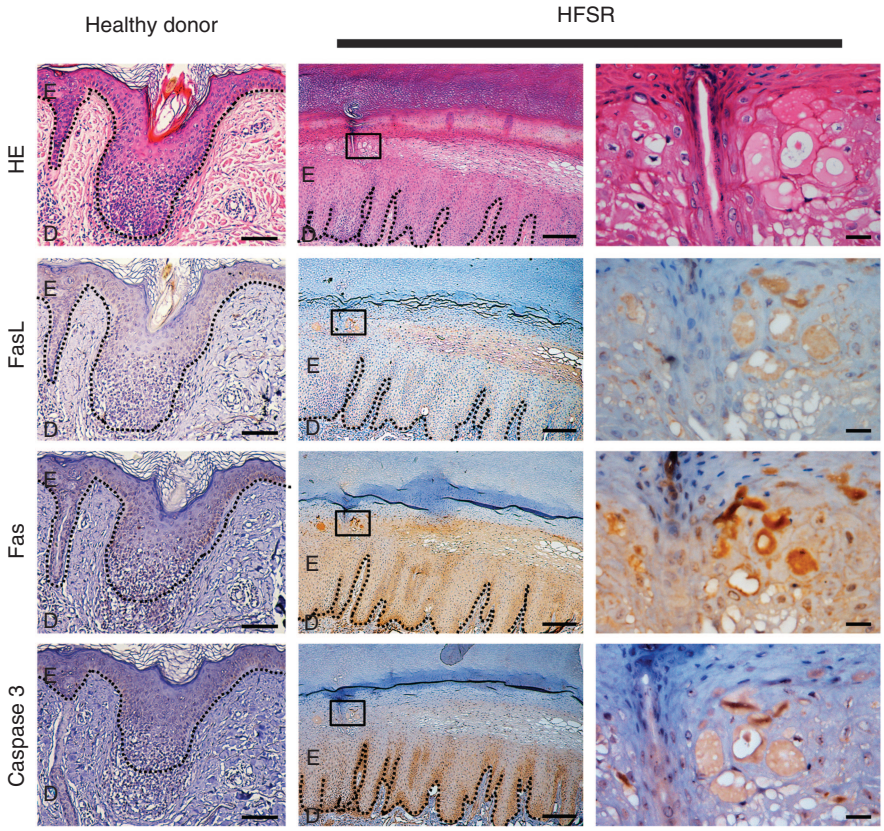

Figure 2. Increased expression of FasL, Fas, and cleaved caspase 3 in the skin lesions of HFSR patients. Skin biopsies were obtained from an affected area in the sole of a patient with grade 3 hand-foot skin reaction (HFSR) or a healthy donor. The skin sections were stained by hematoxylin and eosin, or specific antibodies against FasL, Fas, or active caspase 3. The basement membrane was indicated by a dotted line, and locations of epidermis (E) and dermis (D) were noted. The marked areas were shown in high magnification, in which the increased expression of Fas, FasL, and caspase 3 was noticed around apoptotic keratinocytes within epidermis in the skin biopsies of HFSR. Photographs of one representative HFSR case are shown. Left panels, bar = 100 μm; central panels, bar = 200 μm; right panels, bar = 20 μm.

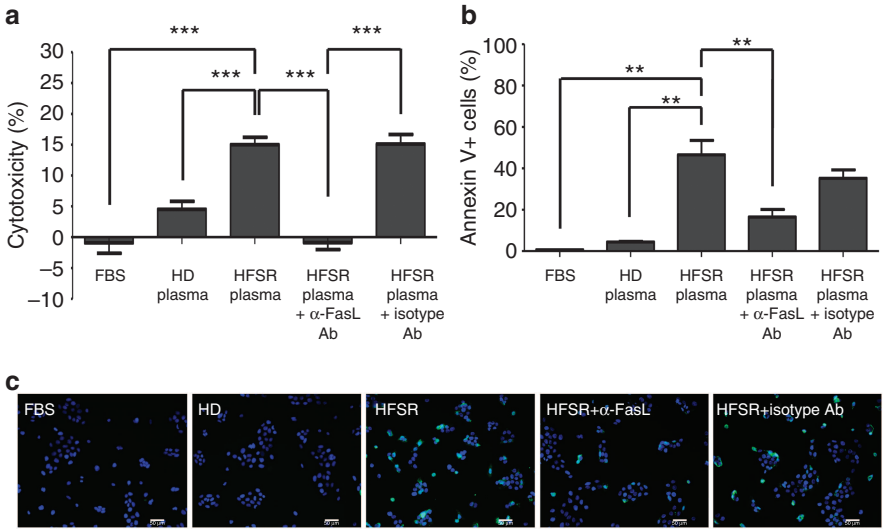

Figure 3. FasL mediates the cytotoxicity of HFSR plasma to keratinocytes. Keratinocytes (HaCaT cells) were cultured in the medium containing 10% fetal bovine serum, 10% plasma from healthy donors (HD; $n = 5$), 10% plasma from hand-foot skin reaction (HFSR) patients ($n = 5$), or 10% HFSR plasma preincubated with an anti-FasL antibody (5G51) or an isotype control antibody. Cytotoxicity (**a**) and apoptosis (**b**) were analyzed by the CCK-8 assay after a 24-hour incubation and by annexin V staining after a 6-hour incubation, respectively. The assays were performed in triplicate, and data are presented as mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$. (**c**) Photographs depicting the early-stage apoptosis of human keratinocytes stained with annexin V (FITC green fluorescence). The blue color represented 4′,6-diamidino-2-phenylindole-stained nuclei of cells. Bar = 50 μm.

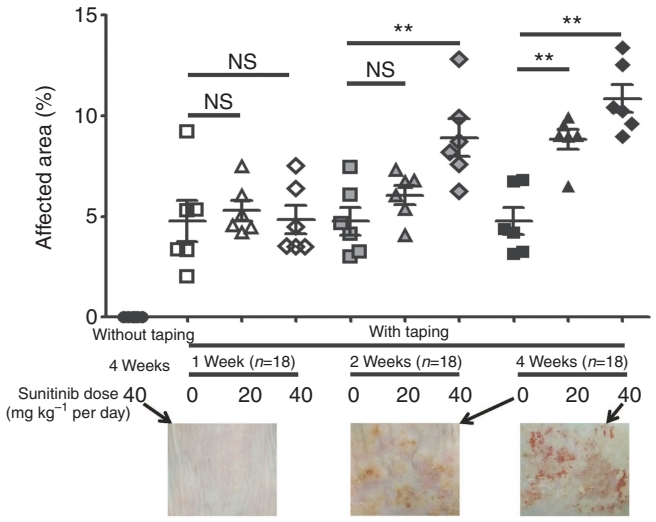

Figure 4. Oral administration of sunitinib to mice increased the skin susceptibility to mechanical injuries. C3H/HeJ mice were assigned to nine groups (each group, $n=6$): three vehicle groups without sunitinib administration serving the controls of 1, 2, and 4 weeks, and six groups given sunitinib daily (20 mg kg^{-1} per day or 40 mg kg^{-1} per day) for 1, 2, and 4 weeks by oral gavage. Tape stripping was applied on the dorsal skin for 4 days from the 4th day to the 7th day of the 1st, 2nd, or 4th week. Photographs of dorsal skin were taken before or after the tape stripping, and the percentage of affected area (measuring the wound area to the taped area) was analyzed by ImageJ as described in the Materials and Methods. Representative photos of the dorsal skin are shown. Results presented are mean ± SEM. NS, no significance; $^{**}P<0.01$.

in mice given sunitinib (40 mg kg^{-1} per day, 4 weeks) and tape-stripping application (Figure 5). Furthermore, the epidermis of the foot pads of mice treated with sunitinib showed increased dyskeratosis in H&E staining, although there was no gross change observed (Figure 5). By immunohistochemistry staining, we found that sunitinib-treated mice showed strong expression of Fas/FasL in the ulcerated epidermis of tape-stripped back skin (Figure 5). Consistently, the increased expression of Fas/FasL was also detected in the epidermis of foot pads of sunitinib-treated mice (Figure 5). In contrast, no or only weak expression of Fas/FasL was observed in the epidermis of vehicle controls (Figure 5). These data further support that Fas/FasL interaction is involved in keratinocyte death in sunitinib-induced HFSR.

DISCUSSION

This study revealed the increased expression of sunitinib and FasL in the plasma of HFSR patients, and their concentrations showed strong correlation. The plasma of HFSR patients caused keratinocyte death, and such cytotoxicity could be blocked specifically by an anti-FasL antibody. Oral administration of sunitinib to mice increased the skin susceptibility to mechanical pressure. Mice administered sunitinib at 40 mg kg^{-1} per day for 4 weeks showed the maximally affected skin area with erosion-to-ulceration response to tape-stripping application. Furthermore, increased expression of FasL, Fas, and active caspase 3 was detected in the epidermis of skin lesions in HFSR patients as well as in the tape-stripped skin or foot pads of mice treated with sunitinib. These findings suggest a pathogenic role of Fas/FasL interaction in keratinocyte death in sunitinib-induced HFSR, and shed light on the molecular mechanism of HFSR.

The Fas/FasL system is a key molecular regulator of apoptosis. There is evidence revealing that dysregulation of FasL expression and/or signaling contributes to the pathogenesis of various cutaneous diseases, such as toxic epidermal necrolysis (Viard *et al.*, 1998; Viard-Leveugle *et al.*, 2013), fixed drug eruption (Choi *et al.*, 2006), acute graft versus host disease (Baker *et al.*, 1996), maculopapular rashes (Wang *et al.*, 2011), lichenoid dermatoses (Farley *et al.*, 2011), and Behcet's syndrome (Baris *et al.*, 2005). In addition, FasL signaling has been shown to be functional in human keratinocytes (Sayama *et al.*, 1994). Under basal conditions, keratinocyte FasL is expressed at very low levels and is not cytolytic because of its intracellular localization (Viard-Leveugle *et al.*, 2003). Upon appropriate stimulation, however, keratinocyte FasL can be induced to become lytic to cells (Gutierrez-Steil *et al.*, 1998).

Through screening 29 various cytokines and cytotoxic proteins, we identified a significant elevation of sFasL in the plasma of HFSR patients. The increase of sFasL does not seem to be derived from peripheral blood mononuclear cells as there was no difference in the expression level of FasL in peripheral blood mononuclear cells from sunitinib-induced HFSR and tolerant controls (Supplementary Figure S5 online). In addition, the plasma sFasL was not contributed by the cancer tissues, as no increased expression of FasL was detected in GIST cell lines or tumor specimens after sunitinib treatment (Supplementary Figure S6 online). Our histological data showing keratinocyte apoptosis accompanied with the increased Fas and FasL expression in the epidermis of HFSR patients and the mouse model imply that elevated sFasL detected in the plasma, in part, may result from cleavage of a membrane-bound FasL on the keratinocytes of HFSR patients.

It is intriguing that sunitinib treatment made the skin more susceptible to physical damage and such injury was associated with increased expression of FasL in keratinocytes. We observed the strong correlation between the plasma levels of sunitinib and FasL in HFSR patients. The direct cytotoxicity of FasL on the keratinocytes and colocalization of FasL, Fas, and active caspase 3 in the epidermis of HFSR patients and sunitinib-treated mouse models indicate the pathogenic role of sFasL in HFSR. Moreover, the increased mouse susceptibility to mechanical injury and induction of keratinocyte FasL/Fas in the animal experiments further support the combined effects of sunitinib toxicity and physical pressures in HFSR. Further studies are needed to investigate how poor metabolism of Y kinase inhibitors and mechanical/physical pressure interplay with Fas/FasL signaling and cause keratinocyte death in HFSR.

In addition to a key player of apoptosis, different functions of FasL in regulation of cell activation and angiogenesis have been proposed (Alderson *et al.*, 1993; Biancone *et al.*, 1997). Furthermore, Fas/FasL interactions have been suggested to be proinflammatory, because Fas signaling results in the activation of caspases that cleave and activate the proinflammatory

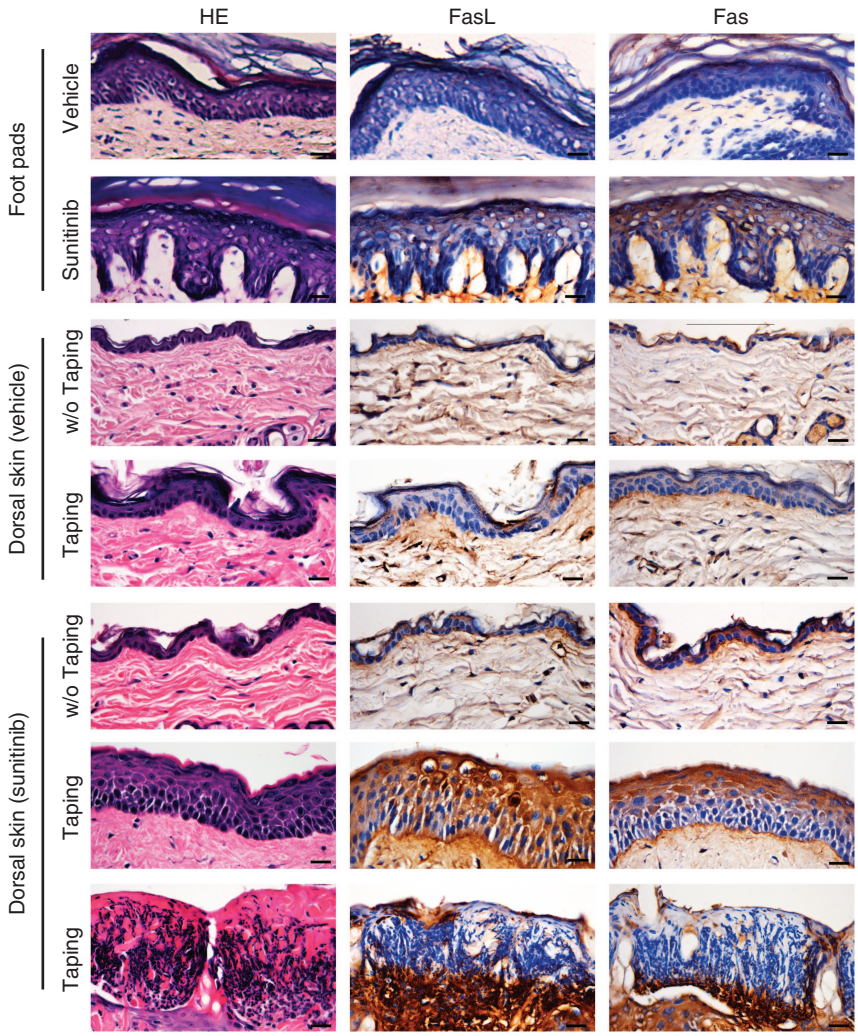

Figure 5. Sunitinib increased the expression of Fas/FasL in the keratinocytes of epidermis in mice. The skin biopsies of dorsal skin or foot pads were obtained from vehicle controls or mice given sunitinib (40 mg kg^{-1} per day) for 4 weeks. Tape stripping was applied on the dorsal skin for 4 days from the 4th day of the 4th week. At the end of the 4th week, the dorsal skin (with taping or without (w/o) taping) and foot pads (without taping) were excised and the skin biopsies were subjected to hematoxylin and eosin (HE) staining and immunohistochemistry studies using specific antibodies against FasL or Fas. Representative photographs are shown. Bar = 20 μm.

cytokines (Restifo, 2000). Also, it has been documented that FasL production, in particular membrane-bound FasL, is effectively linked to inflammation (Hohlbaum *et al.*, 2000). In this regard, the Fas/FasL system may also be potentially implicated in the inflammation in HFSR along with apoptosis. This suggests that the dysregulation of the FasL signaling pathway may not only contribute to the apoptosis of keratinocytes but also likely promote the following inflammatory responses, leading to the extensive skin damage in HFSR.

In conclusion, this study demonstrated that the increased FasL expression may trigger keratinocyte apoptosis in HFSR caused by sunitinib treatment, indicating a pathogenic role for Fas/FasL signaling in HFSR. Our findings provide insights into the molecular basis of HFSR and may shed additional light on the development of better management techniques for HFSR.

MATERIALS AND METHODS

Study participants

From 2006 to 2012, we recruited 53 patients with immunohistologically confirmed metastatic GIST, who were treated with sunitinib at Chang Gung Memorial Hospital (CGMH), Taiwan. This study was conducted according to the Declaration of Helsinki Principles, and approved by the institutional review board of CGMH. Written informed consent was obtained from each participant. Patients were administered 50 mg (4 weeks on, 2 weeks off) or 37.5 mg (continuously) of sunitinib in 12.5 mg capsules taken orally daily with food. The toxic effects were recorded in accordance with the National Cancer Institute Common Toxicity Criteria. Blister cells/fluids were obtained from patients with grade 3 HFSR or from subjects with burn injuries. Plasma samples were obtained from the acute stage of HFSR patients, or at the end of a 4-week administration of daily dosing sunitinib in tolerant controls. The plasma levels of sunitinib were

determined by the high-performance liquid chromatographic method (Blanchet *et al.*, 2009).

ELISA and Bio-Plex

The expression levels of 27 cytokines, granulysin, and FasL in the plasma samples or blister fluids were measured using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad Laboratories, Hercules, CA) or ELISA (R&D Systems, Minneapolis, MN). The 27 cytokines include IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, tumor necrosis factor-α-α, and VEGF.

Animal study protocol

The Experimental Animal Ethics Committee of the institute approved the animal protocols of this study. This investigation conformed to the US National Institute of Health (NIH) guidelines for the care and use of laboratory animals (Publication No. 85-23, revised 1996). To establish an animal model of HFSR, we orally administered different doses of sunitinib (0, 20, 40 mg kg^{-1} per day) to C3H/HeJ mice (4-week-old females) for various time periods (1, 2, and 4 weeks). The animals were randomized into nine groups (each group, $n=6$): three vehicle groups without sunitinib administration serving the controls of 1, 2, and 4 weeks, and six groups receiving sunitinib daily (20 mg kg^{-1} per day or 40 mg kg^{-1} per day) for 1, 2, and 4 weeks by oral gavage. The backs of mice were shaved to observe the skin change. To create mechanical pressure on the mouse skin, we adapted the previously published method (Sano *et al.*, 2005) and performed tape stripping using 20 strokes with transparent tape on the dorsal skin every day for 4 days from the fourth day of the 1st, 2nd, and 4th week. Images were taken before and after tape stripping, and tissues were collected after the course of procedure. At the 7th day of the 1st, 2nd, and 4th week, the animals were anesthetized using pentobarbital (15 mg kg^{-1}, intraperitoneally), and the back skin and foot pads were excised and fixed in 4% paraformaldehyde for histopathological and immunohistochemical analyses. The photographs of skin injuries were analyzed by Bernsen's auto local thresholding method v1.4 using ImageJ 1.47 m. The skin injury was calculated as the ratio of the wound area to the tape-stripped area and expressed as the percentage of the affected area.

Histopathological and immunohistochemical staining

Mice. H&E staining and immunohistochemical analyses were performed on snap-frozen, OCT Tissue-Tek (Sakura Finetek, Torrance, CA) cryoprotectant-embedded, paraformaldehyde-fixed mouse skin tissue. For immunohistochemical staining, the slides of skin tissues from the back or foot pads were incubated with the primary antibodies against FasL (#ab68338, Abcam, Cambridge, MA), Fas (#ab82419, Abcam), or phospho-PDGF Receptor β (Tyr1021) (#ab16868, Abcam) overnight at 4 °C, washed three times for 5 min in tris-buffered saline with tween 20, and incubated with the secondary antibody before visualization with the DAKO LSAB2 system peroxidase (DAKO A/S, No K0675, Carpentaria, CA). The control slides were incubated with the secondary antibody, or isotype control antibodies alone.

Patients. Skin specimens were obtained from the affected area of foot of patients with grade 3 HFSR or healthy subjects and stained with H&E. The specimens were stained with primary antibodies (1:200 dilution) against FasL (NCL-Fas-L, Leica Biosystems, St Louis, MO), Fas (NCL-FAS-310, Leica Biosystems), or cleaved caspase 3 (#9664S, Cell Signaling, Danvers, MA) overnight at 4 °C. The control slides were incubated with the secondary antibody or isotype control antibodies and then washed three times (5 min each) in tris-buffered saline with tween 20 and incubated with the secondary antibody before visualization using the DAKO LSAB2 system peroxidase (DAKO A/S, Copenhagen, Denmark, No K0675). After washing, the slides were mounted and microscopically analyzed by the investigators, who were unaware of the source of the slides.

Cell culture and cytotoxicity assay

HaCaT human keratinocytes were obtained from ATCC. HaCaT cells were cultured in DMEM in 96-well plates at the density of 2×10^4 cells per well with 10% plasma from sunitinib-induced HFSR patient, 10% plasma from healthy donors, or 10% fetal bovine serum. For blocking the Fas/FasL interaction, a FasL antibody (ALX-804-231-C100, clone: 5G51, Enzo Life Sciences, Farmingdale, NY) or isotype control (6602872, Beckman Coulter, Brea, CA) was added to the culture medium with a final concentration of 0.5 μg ml^{-1} for 1 hour at 37 °C before the incubation with keratinocytes. After 24 hours of incubation, the cell viability was analyzed by the cell counting kit-8 (CCK-8 assay, Sigma, St Louis, MO).

Annexin V staining

HaCaT cells were incubated on collagen-coated 8-well chamber slides (Nunc, Thermo Scientific, Waltham, MA) at a density of 4×10^4 cells per well overnight and treated with the indicated conditions for 6 hours. After the treatment, cells were washed by phosphate-buffered saline, fixed with 4% paraformaldehyde, and then incubated with anti-annexin V-FITC (eBioscience, San Diego, CA) and 4′,6-diamidino-2-phenylindole (Sigma). For immunofluorescence imaging, photographs were taken under a Nikon (Tokyo, Japan) Eclipse TE2000U fluorescence inverted microscope equipped with a CCD camera and MetaMorph software (Universal Imaging, Bedford Hills, NY). Apoptotic response was calculated as the ratio of the number of cells immunostained for membrane annexin V to the total number ;of nuclei per field and expressed as the percentage of annexin V–positive cells.

Statistical analysis

All statistical analyses were performed using the SPSS computer software package (Version 10.0, Chicago, IL). The expression levels of FasL and biomarkers in different groups were compared by nonparametric tests. The plasma levels of sunitinib in HFSR patients or tolerant controls were analyzed by the Mann–Whitney *U*-test. Linear regression was applied to analyze the correlation of plasma levels of FasL and sunitinib in the HFSR patients. All of the *P*-values were two-tailed, and a *P*-value of <0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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